Award Number: W81XWH-12-1-0451

TITLE: Structural Basis of Merlin Tumor Suppressor Functions in Neurofibromatosis-2

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REPORT DATE: October 2013

TYPE OF REPORT: Annual report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2013	Annual report	30September2012-29September2013

4. TITLE AND SUBTITLE
Structural Basis of Merlin Tumor Suppressor Functions in Neurofibromatosis-2

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0451

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, CA 92037-1000

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Loss-of-function mutations in the neurofibromatosis-2 (NF2) gene lead to familial and sporadic neurological malignancies in man, specifically to schwannomas and meningiomas, and also to malignant mesothelioma in the lung. Furthermore, Nf2 heterozygosity in mice leads to the development of a number of aggressive tumors, indicating that merlin, the cytoskeletal protein encoded by the NF2 gene, plays broad roles in harnessing tumorigenesis, which are essentially incurable and the overall outcome of Neurofibromatosis Type II (NF2) patients with these cancers remains dismal, despite aggressive therapy

15. SUBJECT TERMS- none provided

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Loss-of-function mutations in the *neurofibromatosis-2 (NF2)* gene lead to familial and sporadic neurological malignancies in man, specifically to schwannomas and meningiomas, and also to malignant mesothelioma in the lung. Furthermore, *Nf2* heterozygosity in mice leads to the development of a number of aggressive tumors, indicating that merlin, the cytoskeletal protein encoded by the *NF2* gene, plays broad roles in harnessing tumorigenesis, which are essentially incurable and the overall outcome of Neurofibromatosis Type II (NF2) patients with these cancers remains dismal, despite aggressive therapy. Loss of function mutations in merlin disables cell-cell junctions in primary Schwann cells, the principle target cell where cancer arises in NF2 patients. As a consequence of merlin loss, *NF2*-deficient cells display uncontrolled growth, loss of contact inhibition, and alterations in the expression of mitogenic signaling cell surface receptors. Notably, all of these phenotypes are suppressed by re-introduction of merlin into human or mouse *Nf2*-deficient Schwann cells, establishing merlin as a *bona fide* tumor suppressor. This DOD Neurofibromatosis New Investigator grant application proposes to define the long-sought crystal structure of the human merlin tumor suppressor, and to define how merlin mutations disrupt its structure and function. The proposed studies of **Aim 1** will complete the crystal structure of the merlin head/tail complex as well as the full-length merlin structure to reveal how tail binding provokes merlin head domain unfurling and dimerization. The studies of **Aim 2** seek to define regulatory control of merlin function *via* heterotypic head-tail interactions with ezrin, which blocks merlin tumor suppressor functions. The effects of head and tail binding of ezrin on merlin structure will be defined, as well as effects on merlin head domain unfurling and dimerization.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report.

In the past year, we focused our efforts on the most challenging and most important aspects of the proposed studies, namely the structure determination of human full-length merlin (**Aim 1**). We also established an expression and purification protocol allowing us to generate large amounts of pure merlin FERM domain (**Aim 1 and 2**).

- Provide data explaining the relationship of the most recent findings with that of previously reported findings.

- Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report.

If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures.

- The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks.
- Statistical tests of significance shall be applied to all data whenever possible.
- Figures and graphs referenced in the text may be embedded in the text or appended.
- Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative.
- This approval must be obtained prior to initiating any change to the original Statement of Work.

The first crucial step towards determining the full-length human high-resolution crystal structure of merlin is the generation of large amounts of homogeneous purified merlin, which often is not a trivial task and in this case is a challenge. Over the past year of funding we explored several expression hosts and constructs with different affinity tags. To keep this report focused, only the key results are shown.

For full-length merlin production, we used several constructs for isoform 1, in particular all residues (1-595), constructs with the disordered *N*-terminus deleted (18-595 or 20-595) and also isoform 2 (residues 20-590). For expression in

Escherichia coli, we used several strains, such as BL21(DE3), Rosetta, Origami, RIL, or RIL(DE3). Best success was accomplished as an MBP-fusion. Merlin did not express in BL21(DE3), Rosetta, or Origami (data not shown) but in RIL, or RIL(DE3). As is evident, amino-terminal truncation is crucial to obtain good expression (**Figure 1**).

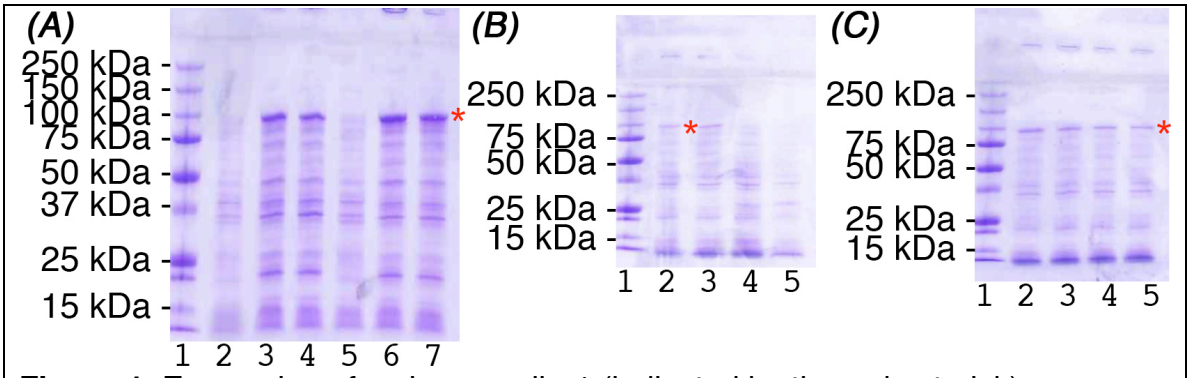

Figure 1. Expression of various merlin-1 (indicated by the red asterisk) constructs in various *Escherichia coli* hosts using a variety of media. **(A)** Expression in RIL(DE3): Merlin 1-595 (lanes 2 and 5), MBP-merlin 18-595 (lanes 3 and 6), and MBP-merlin 20-595 (lanes 4 and 7) using the autoinduction media at 30 °C (lanes 2-4) or induction by 0.4 mM IPTG at 25 °C (lanes 5-7). **(B)** Expression in RIL: Merlin isoform 2 residues 20-590 (lanes 2 and 3) and MBP-merlin1 residues 1-595 (lanes 4 and 5) using the autoinduction media at 30 °C (lanes 2 and 4) or induction by 0.4 mM IPTG at 25 °C (lanes 3 and 5). **(C)** Expression in RIL: Merlin residues 18-595 (lanes 2 and 3) and merlin residues 20-595 (lanes 20-595) using the autoinduction media at 30 °C (lanes 2 and 4) or 0.4 mM IPTG induction at 25 °C (lanes 3 and 5).

After obtaining good expression results (**Figure 1**), we proceeded with protein purification. A large amount of parameters were varied and tested and only the major results are presented which can be summarized in difficulties in purification to homogeneity. Despite a large number of tested chromatography columns, lower molecular weight impurities remain and binding to the amylose affinity column was incomplete (**Figure 2**).

To overcome the expression and purification difficulties described above, we proceeded with merlin (residues 1-595, 18-595, and 20-595) expression in *Pichia pastoris* using standard vectors as well as vectors that secrete recombinant proteins due to an *N*-terminal peptide encoding *Saccharomyces cerevisiae* secretion signal. We generated constructs harboring a *C*-terminal cleavable tag comprising GFP for easy detection of expression followed by ten Histidine residues for easy purification.

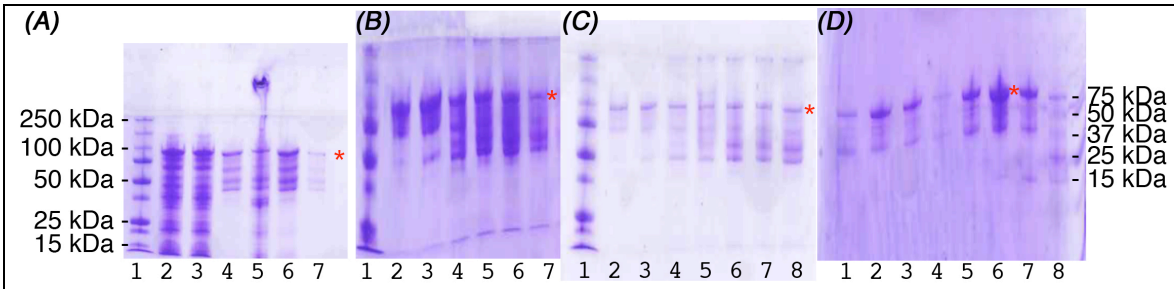

Figure 2. Purification of human merlin (indicated by the red asterisk). **(A)** MBP affinity chromatography: SDS-PAGE of fractions of merlin 18-595 (lanes 2, 3, 5, and 6) and residues 20-595 (lanes 4 and 7) showing the flow through (lanes 2-4) and the elution with maltose (lanes 5-7). **(B)** Anion exchange chromatography: SDS-PAGE of fractions of merlin 18-595 (lanes 2-4) and residues 20-595 (lanes 5-7) from a Q HP anion exchange column. **(C)** Second pass of unbound merlin residues 18-595 lysate back onto MBP affinity chromatography. Fractions are shown in lanes 2-8. **(D)** Second pass of unbound merlin residues 20-595 lysate back onto MBP affinity chromatography. Fractions are shown in lanes 1-7.

We also obtained a construct of merlin residues 1-339 from our colleague in our Department, Dr. Joseph Kissil, as a GST-fusion. Although we were able to obtain good expression in Rosetta, RIL, and RIL(DE3), the expressed protein was insoluble (data not shown). This was eventually overcome by lowering the expression temperature to 25 °C and resulted in pure protein (**Figure 3**) that we will use in our structural studies to obtain the complex for merlin with merlin (**Aim 1.1**) or ezrin (**Aim 2.2**) and for biochemical studies (**Aim 1.3**).

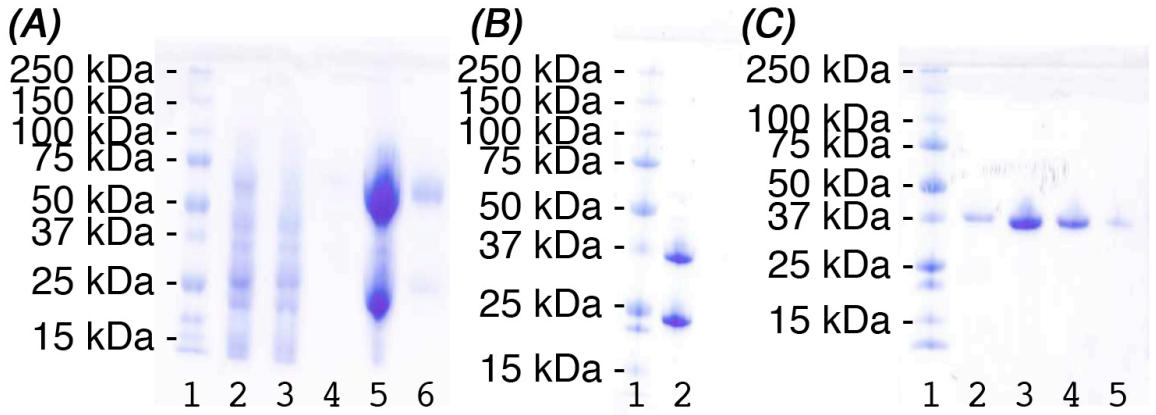

Figure 3. Generation of pure merlin FERM domain. **(A)** SDS-PAGE of fractions from a GST affinity chromatography column. The flow through is shown in lane 3 and the elutants with glutathione in lanes 5 and 6. **(B)** Cleavage with PreScission protease results in two bands (lane 2) for merlin (higher molecular weight band) and GST (lower molecular weight band). **(C)** SDS-PAGE of merlin 1-339 fractions from a size exclusion chromatography column (superdex 75) that separated GST (not shown) from merlin (lanes 2-5).

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

Establishing the expression and purification protocols for full-length human merlin and individual domains will move the neurofibromatosis field forward in fully characterizing the protein responsible for this disease.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- manuscripts, abstracts, presentations; In progress
- licenses applied for and/or issued; Not applicable
- degrees obtained that are supported by this award; Not applicable
- development of cell lines, tissue or serum repositories; Not applicable
- infomatics such as databases and animal models, etc.; Not applicable
- funding applied for based on work supported by this award;
  We submitted a request for funding (entitled “Structure and function of the merlin tumor suppressor in adhesion complexes in Neurofibromatosis Type II”) *via* a DOD Neurofibromatosis Exploration – Hypothesis Development Award grant mechanism where we propose to study the merlin/α-catenin interaction.

- employment or research opportunities applied for and/or received based on experience/training supported by this award. Not currently applicable

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

Establishing the expression and purification protocols for full-length human merlin and individual domains will move the neurofibromatosis field forward in fully characterizing the protein responsible for this disease. We have made significant progress towards this goal during the first year of funding and thus towards determining the long-sought crystal structure of the human merlin tumor suppressor, and to define how merlin mutations disrupt its structure and function.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

Not applicable

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

Not applicable

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.